

(19)



Europäisches Patentamt
European Patent Office
Offic européen des brevets



(11) Publication number:

0 502 476 A2

(12)

EUROPEAN PATENT APPLICATION(21) Application number: **92103610.9**(51) Int. Cl.⁵: **C12N 15/74, C12N 15/70,
C12N 15/55, C12N 1/21,
/(C12N1/21,C12R1:01)**(22) Date of filing: **03.03.92**

The applicant has filed a statement in accordance with Rule 28 (4) EPC (issue of a sample only to an expert). Accession number(s) of the deposit(s): FERM BP-3728, 3729, 3730, 3731, 3732, 3733 and 3734.

(30) Priority: **04.03.91 JP 37544/91
04.03.91 JP 37545/91**(43) Date of publication of application:
09.09.92 Bulletin 92/37(84) Designated Contracting States:
DE FR GB

(71) Applicant: **NITTO CHEMICAL INDUSTRY CO., LTD.**
No. 5-1, Marunouchi 1-chome Chiyoda-ku Tokyo(JP)
Applicant: **Beppu, Teruhiko**
No. 5-21, 1-chome
Horinouchi Suginami-ku, Tokyo(JP)

(72) Inventor: **Beppu, Teruhiko**
5-21 Horinouchi 1-chome, Suginami-ku Tokyo(JP)

Inventor: **Horinouchi, Sueharu**
3-16-403, Etsuchujima 1-chome Koutou-ku, Tokyo(JP)

Inventor: **Nishiyama, Makoto**
16-11, Nishiochiai 2-chome Shinjuku-ku, Tokyo(JP)

Inventor: **Yu, Fujio**
24-4-108, Kirigaoka 3-chome, Midori-ku Yokohama-shi, Kanagawa-ken(JP)

Inventor: **Hashimoto, Yoshihiro**
8-2-104, Yayoi 2-chome, Bunkyo-ku Tokyo(jp)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4 P.O. Box 86 07 67 W-8000 München 86(DE)

(54) **Hybrid plasmid vectors containing genes encoding nitrile degrading enzymes and methods of producing amides and acids.**

(57) The present invention provides a recombinant plasmid comprising combining a hybrid plasmid vector with the isolated DNA sequences of one or more genes encoding nitrile degrading enzymes which are derived from bacteria belonging to the genus *Rhodococcus*, said hybrid plasmid vector comprising an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to the genus *Rhodococcus*, and an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to *Escherichia coli*, and an isolated DNA sequence containing a drug resistance gene.

EP 0 502 476 A2

The present invention relates to novel hybrid plasmid vectors, recombinant plasmids containing a nitrile hydratase gene and an amidase gene, transformants that are made by transforming bacteria belonging to the genus *Rhodococcus* or *Escherichia* with the recombinant plasmids and to methods of producing amides and acids.

Microorganisms belonging to the genus *Rhodococcus* are known to hydrate or hydrolyze nitriles to produce amides or acids (EP Publication No. 0188316, 0204555, and 0348901). In addition, microorganisms belonging to *Rhodococcus rhodochrous* are known to have a high hydration activity of nitriles (EP Publication No. 0307926). These useful properties of the microorganisms have motivated to develop a host-vector system to utilize bacteria belonging to the genus *Rhodococcus*. In reality, vectors suitable to host bacteria belonging to the genus *Rhodococcus* have not been appreciably developed yet. Very few suitable plasmid vectors have been found from *Rhodococcus* sp. H13-A [J.Bacteriol. 170: 638 - 645 (1988)], *Rhodococcus erythropolis* (*rhodochrous*) ATCC12674 [Mol.Gen. Genet., 211:148 - 154 (1988)] and *Rhodococcus rhodochrous* ATCC 4276 which was disclosed by the present inventors in Japanese Patent Application No. 270377/1990. The development of novel vectors which are derived from the genus *Rhodococcus* and industrially useful in culturing microorganisms or in improving the properties of microorganisms, has been awaited.

The present inventors have isolated genes encoding nitrile degrading enzymes from bacteria belonging to the genus *Rhodococcus*, cloned the genes into vectors derived from *Escherichia coli* and investigated the gene expression in *Escherichia coli* hosts. The enzymes having a sufficient enzymatic activity have not been produced in *Escherichia coli* hosts so far [Eur.J.Biochem. 181: 563 - 570 (1989), Biochem.Biophys.Acta., 1088: 225 - 233 (1991)].

Industrially useful, covalently closed circular plasmids derived from bacteria belonging to the genus *Rhodococcus* are potentially suitable plasmids to genetically improve the desirable properties of the genus *Rhodococcus* hosts. These plasmids, however, do not contain drug resistance genes to be used as a marker. Suitable plasmid vectors could be constructed by introducing a marker gene into the plasmids. There is a single example that is described in J. Bacteriol. 170: 638 - 645, 1988.

We have successfully constructed shuttle vectors suitable for industrial use by inserting drug resistance genes to be used as a marker, cloning sites and genes necessary for replication in *Escherichia coli* into covalently closed circular plasmids such as pRC001, pRC002, pRC003 and pRC004. We have subsequently subcloned the gene of a clone encoding a nitrile degradation enzyme into the shuttle vectors to give recombinant plasmids, transformed bacteria belonging to the genus *Rhodococcus* or *Escherichia* with the recombinant plasmids and produced amides and acids using the transformants.

A first invention provides a hybrid plasmid vector comprising

- (A) an isolated DNA sequence which is derived from a plasmid selected from the group consisting of pRC001, pRC002, pRC003 and pRC004 and which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to the genus *Rhodococcus*, and
- (B) an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to *Escherichia Coli*, and
- (C) an isolated DNA sequence containing a drug resistance gene.

A second invention provides a recombinant plasmid comprising combining a hybrid plasmid vector with the isolated DNA sequences of one or more genes encoding nitrile degrading enzymes which are derived from bacteria belonging to the genus *Rhodococcus*, said hybrid plasmid vector comprising
an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to the genus *Rhodococcus*, and
an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to *Escherichia coli*, and
an isolated DNA sequence containing a drug resistance gene.

The hybrid plasmid vectors are useful for culturing microorganisms belonging to the genus *Rhodococcus* or *Escherichia* to be industrially used and for improving the properties of the microorganisms.

The present invention provides transformants that contain a number of genetically engineered recombinant plasmids capable of converting nitriles to amides and acids so that the method of the present invention is more efficient in producing amides and acids than conventional methods.

Fig. 1 shows a restriction map of pRC001, pRC002, pRC003 or pRC004.

Fig. 2 shows a construction of pK1, pK2, pK3, and pK4.

Fig. 3 shows a construction of pA3.

Fig. 4 shows a construction of recombinant plasmid pKRNH2.

Fig. 5 shows a construction of recombinant plasmid pAKR325.

The isolated DNA sequence that confers plasmid vectors the ability to replicate and amplify in the cells of bacteria belonging to the genus *Rhodococcus* may be a whole or a part of plasmid selected from the group consisting of pRC001, pRC002, pRC003 and pRC004.

pRC001, pRC002, pRC003 and pRC004 are derived from *Rhodococcus rhodochrous* ATCC4276, ATCC14349, ATCC14348 and IFO3338, respectively. The restriction map of these plasmids is shown in Fig. 1.

The isolated DNA sequence that confers plasmid vectors the ability to replicate and amplify in the cells of bacteria belonging to *Escherichia coli* may be a whole or a part of plasmid selected from the group consisting of pHSG299, pHSG298, pUC19 and pUC18.

Suitable marker genes which are expressed in hosts such as bacteria belonging to the genus *Rhodococcus* and the genus *Escherichia* and capable of conferring the hosts drug resistance may be a kanamycin resistance gene or an ampicillin resistance gene. Any drug resistance genes or more than one drug resistance gene may be incorporated into plasmid vectors as a marker as long as the presence of the desired plasmid is indicated by the marker.

Host bacteria for the hybrid plasmid vectors of the present invention may be *Rhodococcus rhodochrous* ATCC12674 in the genus *Rhodococcus* and *Escherichia coli* K-12 strains in the genus *Escherichia*. These bacteria can be transformed with the hybrid plasmid vectors.

The recombinant plasmid of the present invention comprises hybrid plasmid vectors such as pK1, pK2, pK3, pK4 and pA3. *Rhodococcus rhodochrous* ATCC12674 was transformed with the hybrid plasmid vectors. The transformants were deposited with Fermentation Research Institute, Agency of Industrial Science and Technology and were assigned the accession number as follows:

	accession number
<i>Rhodococcus rhodochrous</i> ATCC 12674/pK1	FERM BP-3728
<i>Rhodococcus rhodochrous</i> ATCC 12674/pK2	FERM BP-3729
<i>Rhodococcus rhodochrous</i> ATCC 12674/pK3	FERM BP-3730
<i>Rhodococcus rhodochrous</i> ATCC 12674/pK4	FERM BP-3731
<i>Rhodococcus rhodochrous</i> ATCC 12674/pA3	FERM BP-3732

Genes encoding nitrile degradation enzymes may be nitrile hydratase and/or amidase genes.

Any medium components used to grow microorganisms may be used to culture the transformants. Such medium components include carbon sources such as saccharides, e.g., glucose, fructose, sucrose, maltose, organic acids such as acetic acid and citric acid, alcohols such as ethanol and glycerol, natural nitrogen source such as peptone, meat extract, yeast extract, hydrolyzed proteins, and amino acids, and various inorganic or organic ammonium salts and, if necessary, inorganic salts, a trace amount of metal salts, and vitamins.

Microorganisms described above are cultured by methods known in the art: pH 4 - 10, at 20 - 45 °C, aerobic conditions, 10 - 96 hours of incubation. Transformants obtained from culture are used to convert nitrile to amide or acid by the following methods; substrates are added to the suspension of the bacterial cells harvested by centrifugation of the culture; substrates are added to the suspension of the treated bacterial cells (e.g., disrupted bacterial cells, a crude or purified enzyme extract and the like), or to the immobilized bacterial cells by methods known in the art; substrates are added to the culture of the transformants while they grow.

Nitriles used as substrates are represented by the general formula, R-(CN)_n, wherein n is 1 (mononitrile) to more than 2 (polynitrile), R is hydrogen, saturated or unsaturated hydrocarbon residues having various numbers of a carbon atom in a straight chain, a branched chain, or a cyclic form, an amino group, an hydroxyl group, halogen, a carboxyl group, or hydrocarbon residues having substituents. Nitriles include a wide range of compounds, such as acetonitrile, propionitrile, n-butyronitrile, isobutyronitrile, n-valeronitrile, acrylonitrile, methacrylonitrile, benzonitrile, cyanopyridine, malononitrile, succinonitrile, fumaronitrile, chloroacetonitrile, β-hydroxypropionitrile, aminoacetonitrile and β-aminopropionitrile and the like. Reaction is typically carried out by the conditions: concentration of a substrate; 0.1 - 10 (w/v)%, concentration of a bacterial cell density in culture; 0.01 - 10 (w/v)%, pH 4 - 10.

The present invention will be further illustrated by the Examples, which do not limit the scope of the present invention.

Example 1

(1) Construction of Hybrid Plasmid Vectors, pK1, pK2, pK3, and pK4 by Combining pRC001, pRC002, pRC003 or pRC004 with pHSG299

Hybrid plasmid vectors, pK1, pK2, pK3, and pK4, were constructed as is shown in Fig. 2. The DNAs of pRC001, pRC002, pRC003 and pRC004, 1 µg each, were digested with restriction enzyme, ClaI (5 units), at 37°C for one hour. 0.5 µg of pHSG299 DNA (TAKARA SHUZO CO., LTD), which is 2.7kb in size and kanamycin resistant, was digested with 5 units of AccI at 37°C for one hour. After digestion, 1/10 volume of 1M Tris-HCl/pH9.0 was added to both reaction mixtures, which were then dephosphorylated with 1 unit of alkaline phosphatase at 65°C for one hour. The dephosphorylated restriction fragments were electrophoresed along with HindIII digested λ phage DNA as a marker on a 0.7% agarose gel. After electrophoresis, the 2.6kb bands of pRC001, pRC002, pRC003 and pRC004 and the 2.7kb band of pHSG299 were cut out from the gel. DNA was recovered using Gene Clean Kit (FUNAKOSHI K.K.) and dissolved in TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0). Each of pRC001, pRC002, pRC003 or pRC004 DNA solution was combined with an equal amount of a pHSG299 DNA solution. T4 DNA ligase, ATP, dithiothreitol and MgCl₂ were added to the mixtures (final concentration in a mixture: 1 unit of T4 DNA ligase, 1mM ATP, 10 mM dithiothreitol and 10 mM MgCl₂). The ligation mixture was incubated at 4°C overnight.

After incubation, the ligation product was added to the suspension of *Escherichia coli* JM105 competent cells (TAKARA SHUZO CO., LTD) and the mixture was incubated on ice for one hour. The mixture was heat-shocked at 42°C for 2 minutes and a 2xYT medium (0.5% NaCl, 1% yeast, 1.6% tryptone) was added to the mixture. The mixture was then incubated with shaking at 37°C for one hour. After incubation, the mixture was spread on a 2xYT agar plate containing 1mM IPTG (isopropyl-β-galactoside) and 0.02% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The plate was incubated at 37°C overnight. Colonies were grown on the plate. White colonies were picked and grown in 3 ml of a 2xYT medium containing 50 µg/ml of kanamycin at 37°C for 8 hours. After growth, bacterial cells were harvested by centrifugation at 15,000 rpm for 5 minutes. The bacterial cells were then suspended in 0.35 ml of a STET solution (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-HCl, pH8.0). 25 µl of lysozyme (10 mg/ml) was added to the suspension. The suspension was vortexed for 3 seconds, placed in a boiled water bath for 50 seconds and centrifuged at 15,000 rpm for 15 minutes. The supernatant was saved. 0.5 ml of 1:1 TE-saturated phenol/chloroform was added to the supernatant. The mixture was vortexed and then centrifuged at 15,000 rpm for 5 minutes. The top portion was saved. 0.5 ml of diether was added to the top portion and the mixture was vortexed. The mixture was then centrifuged and the top portion was discarded. 0.5 ml of isopropanol and 50 µl of 2.5 M acetic acid soda/pH 4.5 were added to the bottom portion. The mixture was incubated at -80 °C for 30 minutes. After incubation, the mixture was centrifuged at 15,000 rpm for 10 minutes. The pellet was rinsed with 70% ethanol, vacuum dried and resuspended in 0.1 ml of TE buffer. The DNA solution was digested with HindIII, BamHI, SphI, EcoRI and XhoI and a restriction map was constructed. The hybrid plasmids constructed from pRC001, pRC002, pRC003 and pRC004 and pHSG299 were termed pK1, pK2, pK3, and pK4.

These plasmids have identical restriction sites as is shown in Table 1.

Table 1

Hybrid plasmids (pK1, pK2, pK3, pK4)			
Restriction enzyme	Number of sites	Molecular weight (kb)	
EcoRI	1	5.3	5.3
BamHI	2	4.4	1.0
ClaI	1	5.3	5.3
HindIII	2	3.3	2.0
SphI	2	3.6	1.7
XhoI	1	5.3	5.3

(2) Isolation and Purification of Hybrid Plasmids, pK1, pK2, pK3, and pK4

The kanamycin resistance transformants [*Escherichia coli* JM105 (pK1), *Escherichia coli* JM105 (pK2), *Escherichia coli* JM105 (pK3), *Escherichia coli* JM105 (pK4)] were grown in 200 ml of a 2xYT

medium. After growth, bacterial cells were harvested by centrifugation, washed with 40 ml of TES buffer (10 mM Tris-HCl/pH 8.0, 10mM NaCl, 1 mM EDTA) and resuspended in 8 ml of a STET solution (50 mM Tris-HCl/pH8.0, 5 mM EDTA, 35 mM sucrose). 10 mg of lysozyme was added to the suspension and the mixture was shaken at 0°C for 5 minutes. 4 ml of 0.25 M EDTA/pH8.0 was added to the mixture. The mixture was then incubated at 0°C for 5 minutes with occasional gentle swirling. The mixture was then left standing at room temperature. 2 ml of 10% SDS (sodium dodecyl sulfate) and 5 ml of 5 M NaCl were added to the mixture. The mixture was incubated at 4°C for 3 - 12 hours. After incubation, the mixture was centrifuged at 65,000 X g at 4°C for one hour. 4.6 ml of 50% polyethylene glycol (6,000) was added to the supernatant. The mixture was incubated on ice for 3 hours. The mixture was then centrifuged at 1,000 X g for 5 minutes: The pellet was resuspended in 7.5 ml of TES. 8.2 g of CsCl and 0.2 ml of ethidium bromide (15 mg/ml) was added to the suspension. The mixture was subjected to density gradient centrifugation at 130,000 X g for 42 hours. After centrifugation, the plasmid portion was removed under the irradiation of UV light. The plasmid portion was extracted with n-butanol to remove ethidium bromide. The plasmid extract was dialyzed against TE. Plasmid DNA was precipitated with ethanol. The presence of the desired plasmid DNA was confirmed by electrophoresis on a 0.7% agarose gel.

(3) Introduction of Hybrid Plasmids, pK1, pK2, pK3, and pK4 into bacteria belonging to the genus *Rhodococcus*

The bacteria cells of *Rhodococcus rhodochrous* ATCC 12674 at a log phase in growth were harvested by centrifugation, washed 3 times with ice cold sterilized water and resuspended in a 15% PEG(6,000) solution (cell density: more than 10^9 cells/ml). The hybrid plasmid DNAs, 0.01 µg each, were combined with 10 µl of the bacterial cell suspension. The mixture was incubated on ice and then placed in the chamber of a cell fusion apparatus (SHIMAZU Corp., SSH-1). The chamber was cooled and the mixture was subjected to electroporation at a pulse amplitude of 500 µs at an electric field strength of 14kv/cm.

After electroporation, the mixture was incubated at 0°C for 10 minutes and then at 37°C for 5 minutes. After incubation, 1 ml of an MY medium was added to the mixture. The mixture was incubated at 25°C for 3 hours. The mixture was then spread on an MY agar medium plate containing 50 µg/ml of kanamycin. The plate was incubated at 25°C for 3 - 6 days. After incubation, colonies were appeared on the plate. These colonies were spread on an MY agar medium plate containing 50 µg/ml of kanamycin again to confirm that the colonies were kanamycin resistant.

Transformants, each transformed with pK1, pK2, pK3, or pK4, were deposited with Fermentation Research Institute, Agency of Industrial Science and Technology, and were assigned the following accession numbers;

	accession number
<i>Rhodococcus rhodochrous</i> ATCC 12674/pK1	FERM BP-3728
<i>Rhodococcus rhodochrous</i> ATCC 12674/pK2	FERM BP-3729
<i>Rhodococcus rhodochrous</i> ATCC 12674/pK3	FERM BP-3730
<i>Rhodococcus rhodochrous</i> ATCC 12674/pK4	FERM BP-3731

The properties of pK4 are further investigated as described below.

(4) Isolation and Purification of the Hybrid Plasmid Vector from the *Rhodococcus* Transformant

Rhodococcus rhodochrous ATCC 12674/pK4 was grown in 400 ml of an MY medium containing 50 µg/ml of kanamycin. When the OD₆₆₀ of the culture reached at 0.15 - 0.2, 0.5 U/ml of penicillin G was added to the culture. The culture was further incubated until the OD₆₆₀ of the culture reached at 1.0. Bacterial cells were harvested by centrifugation, washed with 40 ml of TES and resuspended in 11 ml of a solution (50 mM of Tris-HCl/pH8.0, 12.5% sucrose, 100mM NaCl, 1mg/ml of lysozyme). The suspension was incubated with shaking at 37°C for 3 hours. 0.6 ml of 0.5 M EDTA, 2.4 ml of 5 M NaCl and 4.4 ml of 4% SDS - 0.7M NaCl were added to the suspension in this order. The mixture was gently swirled and incubated on ice for 18 hours. The mixture was centrifuged at 65,000 X g for at 4°C one hour. After centrifugation, 4.6ml of 50% polyethylene glycol was added to the supernatant. The mixture was incubated on ice for 3 hours. The mixture was then centrifuged at 1,000 X g for 5 minutes. The pellet was resuspended in 5 ml of TES. 7.5 g of CsCl and 2 ml of TES containing 1.5 mg/ml of ethidium bromide were added to the suspension. The mixture was subjected to density gradient centrifugation at 130,000 X g for 42

hours. After centrifugation, plasmid portion was removed under the irradiation of UV light. The plasmid portion was extracted with n-butanol to remove ethidium bromide. The plasmid extract was dialyzed against TE. Plasmid DNA was precipitated with ethanol. The presence of desired plasmid DNA was confirmed by electrophoresis on a 0.7% agarose gel.

(5) Comparison between pK4s isolated from the genus *Escherichia* and *Rhodococcus*

(5)-(i) Molecular Weight

A small amount of the plasmid DNAs was electrophoresed along with markers such as pUC18 (2.69kb), pUC118 (3.16kb), pBR322 (4.36kb) on a 0.7% agarose gel. The size of both plasmid DNAs was about 5.3kb.

(5)-(ii) Restriction Sites

A small amount of the plasmid DNAs was digested with restriction enzymes. The restriction fragments were electrophoresed along with HindIII or PstI digested λ phage DNA as a marker on a 0.7% agarose gel. The band pattern showed that both plasmid DNAs had exactly the same restriction sites as is shown in the Table 1.

(6) Transformation of *Escherichia coli* with pK4 Isolated from *Rhodococcus*

Escherichia coli JM105 was transformed with pK4 isolated from *Rhodococcus rhodochrous* ATCC 12674/pK4. Transformants were screened for kanamycin resistance by a 2xYT agar medium plate containing 50 μ g/ml of kanamycin. Most of them were positive transformants. Plasmid DNA was isolated from 12 of the positive transformants, digested with restriction enzymes and electrophoresed on a agarose gel. The band pattern showed that the isolated plasmid DNA had the same restriction sites as those isolated from pK4 of the genus *Rhodococcus* transformant.

Example 2

Plasmid pA3 was constructed as is shown in Fig. 3. pRC003 DNA was digested with ClaI and the restriction fragment was inserted into the AccI site of pUC19. The construct was designated as pA3. *Escherichia coli* JM105 was transformed with pA3 as described in the Example 1. The transformants were screened by a 2xYT agar medium plate containing 50 μ g/ml of ampicillin, 1mM IPTG and 0.2% X-gal. White colonies were picked and grown. Plasmid DNA was isolated, purified, digested with restriction enzymes and analyzed. *Rhodococcus rhodochrous* ATCC 12674 was transformed with the plasmid DNA. The transformant showed that the plasmid DNA conferred ampicillin resistance (10 μ g/ml of ampicillin) to the host.

The transformant, *Rhodococcus rhodochrous* ATCC 12674/pA3, was deposited with Fermentation Research Institute, Agency of Industrial Science and Technology, and was assigned the accession number FERM BP-3732.

Table 2 shows a restriction enzyme map of pA3.

Table 2

(pA3)			
Restriction enzyme	Number of sites	Molecular weight (kb)	
EcoRI	1		5.3
BamHI	2	4.3,	1.0
ClaI	0		-
HindIII	1		5.3
SphI	2	3.6,	1.7
XhoI	1		5.3

Example 3

(1) Construction of Recombinant Plasmids Containing Nitril Hydratase and Amidase Genes

5 pYUK120 and 121 [Eur. J. Biochem. 181: 563-570 (1989)] or pANH101[Biochim. Biophys. Acta 1088: 225-233 (1991)], which contain a DNA fragment which is originally derived from *Rhodococcus* sp. N-774 and cloned into *Escherichia coli*, comprise a nitrile hydratase gene or an amidase gene, respectively.

(1) - (i) Construction of Recombinant Plasmid pKRNH2

10

pKRNH2 was constructed as is shown in Fig. 4. 1 µg of pYUK120 DNA was digested with SphI and HindIII to give a 4.0kb fragment. 1 µg of pANH101 DNA was digested with PstI and HindIII to give a 4.9kb fragment. 1 µg of pK4 DNA was digested with PstI and EcoRI to give a 2.6kb fragment. 1 µg of pHSG299 DNA was digested with SphI and EcoRI to give a 2.7kb fragment. The digestion reaction was carried out at 15 37° C for one hour. After digestion, the reaction mixture was electrophoresed on a 0.7% agarose gel. These fragments were recovered from the gel and purified by Gene Clean Kit (FUNAKOSHI K.K.). An equal amount of a DNA solution was taken from each purified DNA solution and was combined. T4 DNA ligase was then added to the mixture. The ligation reaction was carried out at 4° C for 15 hours.

After ligation reaction, the ligation product was used to transform *Escherichia coli* JM109. Colonies 20 were screened for white colonies by a 2xYT agar plate containing 25 µg/ml of Kanamycin, 1mM IPTG and 0.02% X gal. Positive colonies were incubated with shaking in a 2xYT medium containing Kanamycin at 37° C for 10 hours. Plasmid DNA was isolated from the bacterial cells, digested with restriction enzymes and analyzed. Plasmid thus obtained is designated as pKRNH2.

25 (1) - (2) Construction of Recombinant Plasmid pAKR325

pAKR325 was constructed as is shown in Fig. 5. DNA fragments were prepared and the DNA fragments were combined. T4 DNA ligase was added to the DNA mixture. After ligation reaction, *Escherichia coli* JM109 was transformed with the ligation product and the transformation mixture was spread on a 2xYT agar 30 plate containing 25 µg/ml of Kanamycin, 25 µg/ml of ampicillin, 1mM IPTG and 0.02% X gal to screen for white colonies. Positive colonies were grown in a 2xYT medium containing ampicillin and Kanamycin. Plasmid DNA was isolated from the bacterial cells, digested with restriction enzymes and analyzed. Plasmid thus obtained is designated as pAKR325.

35 (2) Transformation of Bacteria Belonging to the Genus *Rhodococcus* with pKRNH2 or pAKR325

The bacteria cells of *Rhodococcus rhodochrous* ATCC 12674 at a log phase in growth was centrifuged, washed 3 times with ice cold sterilized water and resuspended in a 15% PEG(6,000) solution (cell density: more than 10⁹ cells/ml). pKRNH2 and pAKR325, 0.01 µg each, were combined with 10 µl of 40 the bacterial cell suspension. The mixture was incubated on ice and then placed in the chamber 11 of a cell fusion apparatus (SHIMAZU Corp., SSH-1). The chamber was cooled and the mixture was subjected to electroporation at a pulse amplitude of 500 µs at an electric field strength of 14kv/cm.

After electroporation, the mixture was incubated on ice for 10 minutes and then at 37° C for 5 minutes. After incubation, 1 ml of an MY medium was added to the mixture. The mixture was incubated at 25° C for 45 3 hours. The mixture was then spread on an MY agar plate containing 50 µg/ml of kanamycin. The plate was incubated at 25° C for 3 - 6 days. After incubation, colonies were appeared on the plate. These colonies were spread on an MY agar medium plate containing 50 µg/ml of kanamycin again to confirm that the colonies were kanamycin resistant.

Transformants, each transformed with pKRNH2 or pAKR325, were deposited with Fermentation Research Institute, Agency of Industrial Science and Technology, and were assigned the following accession 50 numbers;

	accession number
<i>Rhodococcus rhodochrous</i> ATCC 12674/pKRNH2	FERM BP-3733
<i>Rhodococcus rhodochrous</i> ATCC 12674/pAKR325	FERM BP-3734

55

(3) Production of amides Using Transformants

Rhodococcus rhodochrous ATCC 12674/pKRNH2 (hereafter referred to as ATCC 12674/pKRNH2) and *Rhodococcus rhodochrous* ATCC 12674/pAKR325 (hereafter referred to as ATCC 12674/pAKR325) were grown in 10 ml of an MY-glycerol medium (1% glycerol, 0.5% polypeptone, 0.3% yeast extract, 0.3% malt extract) containing kanamycin (50 µg/ml) under the fluorescent light at 25°C for 15 - 72 hours. *Rhodococcus rhodochrous* ATCC 12674/pK4 (hereafter referred to as ATCC 12674/pK4) was used as a control. ATCC 12674/pKRNH2, ATCC 12674/pAKR325 and the control were also grown in the same medium as described above plus 0.1% isobutyronitrile + 0.1% isobutylamide as an inducer for nitrile hydratase under the same culture conditions. Bacterial cells were harvested by centrifugation, washed with 50 mM phosphate buffer/pH7.7 and resuspended in 1 ml of 50 mM phosphate buffer/pH7.7. The suspension was placed on ice under the fluorescent light for one hour. 100 µl of the suspension and 0.8 ml of 50 mM phosphate buffer were combined and the mixture was then incubated at 20°C for 10 minutes. After incubation, 10 µl of 1 M acrylonitrile was added to the mixture, which was then incubated for 30 minutes. 200 µl of 1N-HCl was added to the mixture to stop the reaction. Nitrile hydratase activity was determined by measuring an amount of acrylonitrile and acrylamide in the reaction mixture using gas chromatography. Table 3 shows the nitrile hydratase activity of the transformants and the control. The specific activity was remarkably increased by the addition of the inducer.

Table 3

Nitrile hydratase activity					
Bacterial Strain/Plasmid	Time (hr)	Without inducer		With inducer	
		Growth	Specific	Growth	Specific
		(OD ₆₃₀)	Activity (U/mg cell)	(OD ₆₃₀)	Activity (U/mg cell)
ATCC 12673 /pK4	15	1.84	0.00	1.70	0.00
	24	4.66	0.05	2.27	0.13
	48	4.56	0.10	4.30	1.15
	72	4.11	0.03	6.50	0.06
ATCC 12674 /pKRNH2	15	0.13	0.60	0.10	0.20
	24	0.20	1.28	0.05	25.00
	48	0.25	1.60	0.07	46.60
	72	1.50	0.12	0.32	0.44
ATCC 12674 /pAKR325	15	0.33	0.02	1.09	0.05
	24	2.65	0.08	2.08	4.25
	48	4.85	0.13	3.15	9.01
	72	3.18	0.03	5.04	0.09

(4) Nitrile Hydratase Activities of the Enzyme Encoded by the Gene of Recombinant Plasmid or of Chromosome

(4) - (i) SDS-gel Electrophoresis and Western-blotting

Bacterial cells harvested from 10 ml of culture were washed with phosphate buffer and suspended in 1 ml of phosphate buffer. The bacterial cells were sonicated with keeping the tube containing the cells on ice and the disrupted cells were centrifuged at 15,000 rpm for 10 minutes. The supernatant containing a crude extract was used for the experiment described below. A 12.5% SDS-gel was prepared as follows. 15 ml of solution A (29.2 g of acrylamide, 0.8g/100ml of N,N'-methylenebisacrylamide), 9 ml of solution B (1.5M Tris-HCl/pH 8.8, 0.4% SDS) and 12 ml of distilled water were combined and the separating gel solution was subjected to degassing by an aspirator. 140 µl of 10% ammonium persulfate and 12 µl of TEMED (N,N,N',N'-tetramethylethylenediamine) were added to the resolving gel solution and then the separating gel solution was poured into the gap between the glass plates. Water saturated n-butanol was gently overlaid

onto the surface of the gel solution and the gel was left standing until it was polymerized. In the meantime, a stacking gel solution was prepared. 1.8 ml of solution A, 3 ml of solution C (0.5 M Tris-HCl/pH 6.8, 0.4% SDS) and 7.2 ml of distilled water were combined and the stacking gel solution was subjected to degassing. 36 μ l of 10% ammonium persulfate and 12 μ l of TEMED (N,N,N',N'-tetramethylethylenediamine) were added to the stacking gel solution and the stacking gel solution was mixed well. When the resolving gel solution was polymerized, the n-butanol overlay was gently discarded. The stacking gel solution was poured directly onto the resolving gel and then a comb was inserted into the stacking gel solution. When the stacking gel solution was polymerized, the gel was placed in an electrophoresis apparatus. Both upper and lower buffer reservoirs were filled with electrophoresis buffer (0.025M Tris-HCl, 0.192M glycine, 0.1% SDS). 1/3 volume of sample buffer (0.25 M Tris-HCl, 4% mercaptoethanol, 8% SDS, 40% glycerol) was added to the crude extract (20 μ g of protein). The mixture was heated at 90 °C for 5 minutes and then placed in the wells of the plate. Electrophoresis was carried out at 10 mA for 12 - 15 hours. After electrophoresis, half of the gel was stained to detect proteins and another half was used for Western blotting.

Coomassie Brilliant Blue (CBB) staining: The gel was immersed in a staining solution (0.25% CBB in a 5:1:5 water/acetic acid/methanol solution) and shaken at room temperature for one hour. The staining solution was decanted. The gel was rinsed with water briefly and washed in a 8:1:1 water/methanol/acetic acid solution at room temperature for 24 hours.

Western blotting: Antibody of a rabbit serum specific to nitrile hydratase purified from *Rhodococcus* sp. N-774 was used as a primary antibody.

The proteins on the gel were transferred to a transfer membrane [Poly(vinylidene difluoride), Millipore] using zaltoblot 2-SM17556. The transfer was carried out at 4 mA/cm² for 15 minutes. The transfer membrane was soaked in 100 ml of TBS containing 3 g of heat dissolved gelatine. The membrane was incubated with shaking at room temperature for one hour. The membrane was then transferred to 100 ml of TBS solution containing 1% gelatine. 20 μ l of the primary antibody was added to the solution and the membrane was incubated with shaking at room temperature for 2 hours. After incubation, the membrane was washed 2 times with TBS and then placed in 100 ml of TBS containing 1% gelatine. 50 μ l of a secondary antibody, goat anti-rabbit IgG horseradish peroxidase (GAR-HRP)[Bio-Rad], was added to the TBS. The membrane was incubated with shaking at room temperature for 2 hours. The membrane was washed with distilled water and then 2 times with TBS. The membrane was placed in 100 ml of TBS containing 60 μ l of H₂O₂ in a vessel. 60 mg of a HRP color development reagent (Bio-Rad) dissolved in 20 ml of ice cold methanol was added to the vessel. When the band appeared on the membrane, the membrane was washed with distilled water and dried.

Almost no nitrile hydratase band was found on the gel stained by CBB. However in the ATCC12674/pKRNH2 lane and the ATCC12674/pAKR325 lane on the gel of Western blotting, bands resulting from the binding of the antibody to nitrile hydratase were found at the same position as the one of the band of nitrile hydratase obtained from *Rhodococcus* sp. N-774. In contrast, there was no band found in the control lane (*Rhodococcus rhodochrous*/pK4). ATCC12674/pKRNH2 and ATCC12674/pAKR325 were found to express the nitrile hydratase gene derived from *Rhodococcus* sp. N-774.

(4) - (ii) Photoactivation of Nitrile Hydratase

Nitrile hydratase of *Rhodococcus* sp. N-774 is known to be activated by light illumination while the photoactivation of the nitrile hydratase of *Rhodococcus rhodochrous* ATCC 12674 is not known. It is possible to confirm that whether ATCC12674/pKRNH2 and ATCC12674/pAKR325 express the nitrile hydratase gene derived from *Rhodococcus* sp. N-774 by the photoactivation.

10 ml of an MY-glycerol medium was placed in a test tube and the tube was autoclaved. ATCC12674/pK4, ATCC12674/pKRNH2 and ATCC12674/pAKR325 were placed in a separate tube and grown without exposing to light (the tube was wrapped by aluminum foil) or under the exposure to light. Both cultures were incubated with shaking at 26 °C for 36 hours. After incubation, the nitrile hydratase activity was determined. The bacterial cells grown under dark conditions were kept manipulating only in dark conditions: centrifugation was carried out under a weak red light in the darkroom. ATCC12674/pK4 (control) did not show a photoactivation while ATCC12674/pKRNH2 and ATCC12674/pAKR325 increased an activity, under the exposure to light. The result suggested that ATCC12674/pKRNH2 and ATCC12674/pAKR325 produced an enzyme having a photoactivation shown by *Rhodococcus* sp. N-774.

Table 3

Bacterial Strain/Plasmid	Photoactivation of Nitrile hydratase			
	Dark conditions		Light conditions	
	Growth (OD ₆₃₀)	Specific Activity (U/mg cell)	Growth (OD ₆₃₀)	Specific Activity (U/mg cell)
ATCC 12674 /pK4	9.0	1.2	6.5	1.4
ATCC 12674 /pKRNH2	0.08	96.8	0.18	193
ATCC 12674 /pAKR325	14.5	0.3	13.8	1.7

Example 4

Production of Amides Using Transformants

Rhodococcus rhodochrous ATCC 12674/pKRNH2 (hereafter referred to as ATCC 12674/pKRNH2) was grown in 10 ml of an MYP medium (1% glycerol, 0.5% polypeptone, 0.3% yeast extract, 0.3% malt extract, 0.05% potassium dihydrogenphosphate, 0.05% dipotassium hydrogenphosphate, 50 µg/ml of kanamycin) in the presence of a given amount of methacrylamide, an inducer for nitrile hydratase, or in the absence of the inducer agent, under the fluorescent light at 25° C for 24 - 48 hours. *Rhodococcus rhodochrous* ATCC 12674/pK4 (hereafter referred to as ATCC 12674/pK4) used as a control was also grown under the same culture conditions. Bacterial cells were harvested by centrifugation, washed with 50 mM phosphate buffer/pH7.7 and resuspended in 1 ml of 50 mM phosphate buffer/pH7.7. The suspension was placed on ice under the fluorescent light for one hour. 100 µl of the suspension and 0.8 ml of 50 mM phosphate buffer were combined and the mixture was then incubated at 20° C for 10 minutes. After incubation, 10 µl of 1 M acrylonitrile was added to the mixture, which was then incubated for 10 minutes. 200 µl of 1N-HCl was added to the mixture to stop the reaction. A nitrile hydratase activity was determined by measuring an amount of acrylonitrile and acrylamide in the reaction mixture using gas chromatography. Table 5 shows the nitrile hydratase activity of ATCC12674/pKRNH2 and the control.

Table 5 Nitrile hydratase activity

Bacterial Strain/ Plasmid	Methacrylamide	Growth (OD ₆₃₀)	Specific Activity (U/mg cell)
ATCC 12673 /pK4	0 0.1 0.2	3.2 3.1 3.6	trace 3.2 9.1
ATCC 12674 /pKRNH2	0 0.1 0.2	2.7 2.5 2.1	12.1 45.0 152.3

Example 5

Production of Acids Using Transformants

Rhodococcus rhodochrous ATCC 12674/pKRNH2 (hereafter referred to as ATCC 12674/pKRNH2) was grown in 10 ml of an MYP medium (1% glycerol, 0.5% polypeptone, 0.3% yeast extract, 0.3% malt extract, 0.05% potassium dihydrogenphosphate, 0.05% dipotassium hydrogenphosphate 50 µg/ml of kanamycin) in

the presence of a given amount of methacrylamide, an inducer for amidase, or in the absence of the inducer, under the fluorescent light at 25°C for 24 - 48 hours. *Rhodococcus rhodochrous* ATCC 12674/pK4 (hereafter referred to as ATCC 12674/pK4) used as a control was also grown under the same culture conditions. Bacterial cells were harvested by centrifugation, washed with 50 mM phosphate buffer/pH7.7 and resuspended in 1 ml of 50 mM phosphate buffer/pH7.7. The suspension was incubated on ice under the fluorescent light for one hour. 100 µl of the suspension and 0.8 ml of 50 mM phosphate buffer were combined and the mixture was then incubated at 20°C for 10 minutes. After incubation, 100 µl of 1 M propionamide was added to the mixture, which was then incubated for 1 hour. 200 µl of 1N-HCl was added to the mixture to stop the reaction. An amidase activity was determined by measuring an amount of propionamide and propionic acid in the reaction mixture using gas chromatography. Table 6 shows the amidase activity of ATCC12674/pKRNH2 and the control.

Table 6

Bacterial Strain/Plasmid	amidase activity		
	Methacrylamide	Growth (OD ₆₃₀)	Specific Activity (U/mg cell)
ATCC 12673 /pK4	0	3.2	0.03
	0.1	3.1	0.04
	0.2	3.6	0.05
ATCC 12674 /pKRNH2	0	2.7	0.10
	0.1	2.5	0.50
	0.2	2.1	1.49

Claims

1. A hybrid plasmid vector comprising

(A) an isolated DNA sequence which is derived from a plasmid selected from the group consisting of pRC001, pRC002, pRC003 and pRC004 and which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to the genus *Rhodococcus*, and

(B) an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to *Escherichia coli*, and

(C) an isolated DNA sequence containing a drug resistance gene.

2. The hybrid plasmid vector of claim 1 in which the isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to *Escherichia coli* and which is isolated from a plasmid selected from the group consisting of pHSG299, pHSG298, pUC19 and pUC18.

3. The hybrid plasmid vector of claim 1 comprising pK1, pK2, pK3, pK4 and pA3.

4. A transformant comprising bacteria transformed with any one of hybrid plasmid vectors of claim 1 - 3, said bacteria belonging to the genus *Rhodococcus* or *Escherichia*.

5. A transformant of claim 4 in which bacteria belonging to the genus *Rhodococcus* comprise *Rhodococcus rhodochrous* ATCC 12674.

6. A transformant of claim 4 in which bacteria belonging to the genus *Escherichia* comprise *Escherichia coli* K-12.

7. A recombinant plasmid comprising combining a hybrid plasmid vector with the isolated DNA sequences of one or more genes encoding nitrile degrading enzymes which are derived from bacteria belonging to the genus *Rhodococcus*, said hybrid plasmid vector comprising an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to the genus *Rhodococcus*, and an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to *Escherichia coli*, and an isolated DNA sequence containing a drug resistance gene.

8. The recombinant plasmid of claim 7 in which the hybrid plasmid vector comprises the vector of claim 1.
- 5 9. The recombinant plasmid of claim 7 in which the hybrid plasmid vector comprises the vector of claim 3.
10. The recombinant plasmid of claim 7 in which the genes encoding nitrile degrading enzymes comprises nitrile hydratase and amidase genes.
- 10 11. Microorganisms belonging to the genus *Rhodococcus* that are transformed with any one of the recombinant plasmids of claim 7 - 10.
12. A method of producing amides which comprises hydrating nitriles using the transformant of claim 11.
- 15 13. A method of producing acids which comprises hydrolyzing nitriles using the transformant of claim 11.

20

25

30

35

40

45

50

55

Fig.1

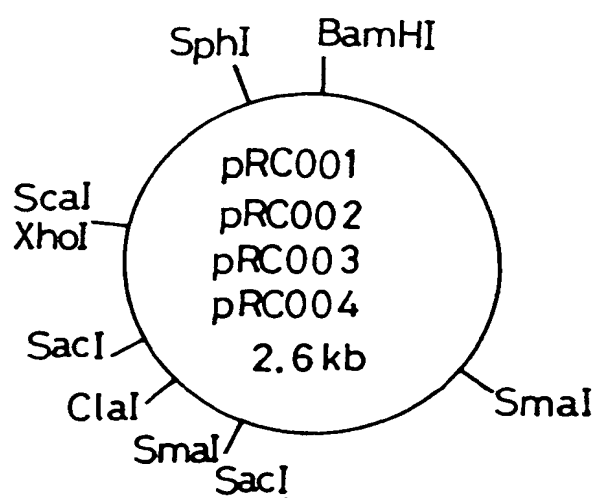


Fig. 2

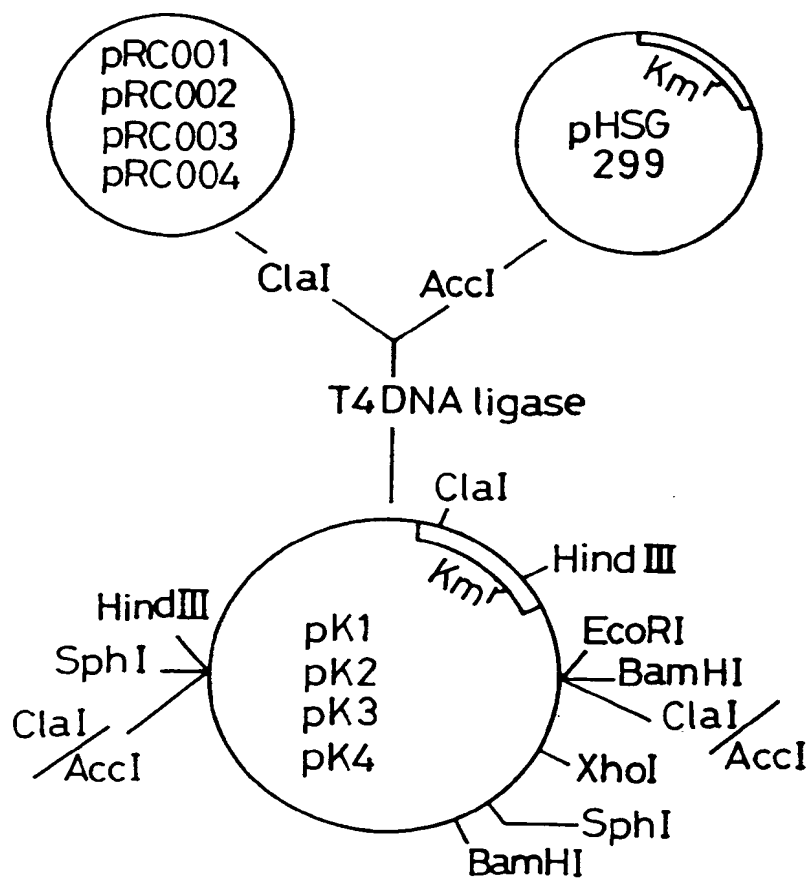


Fig.3

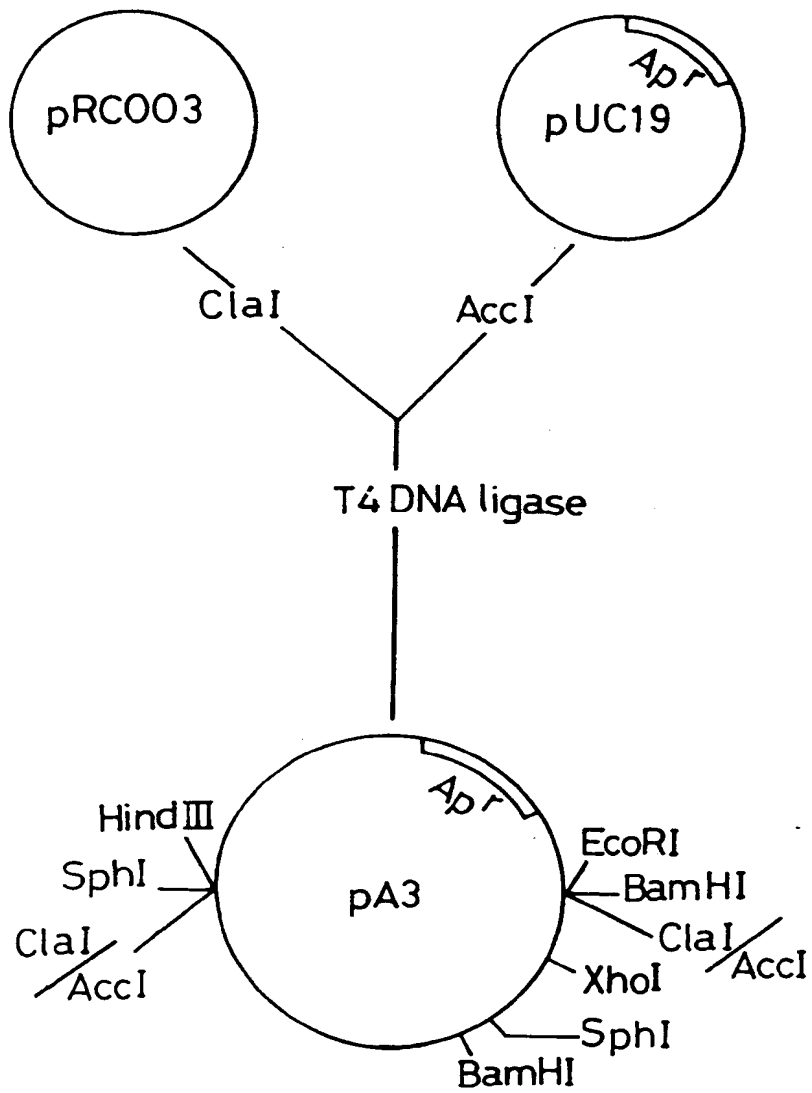


Fig. 4

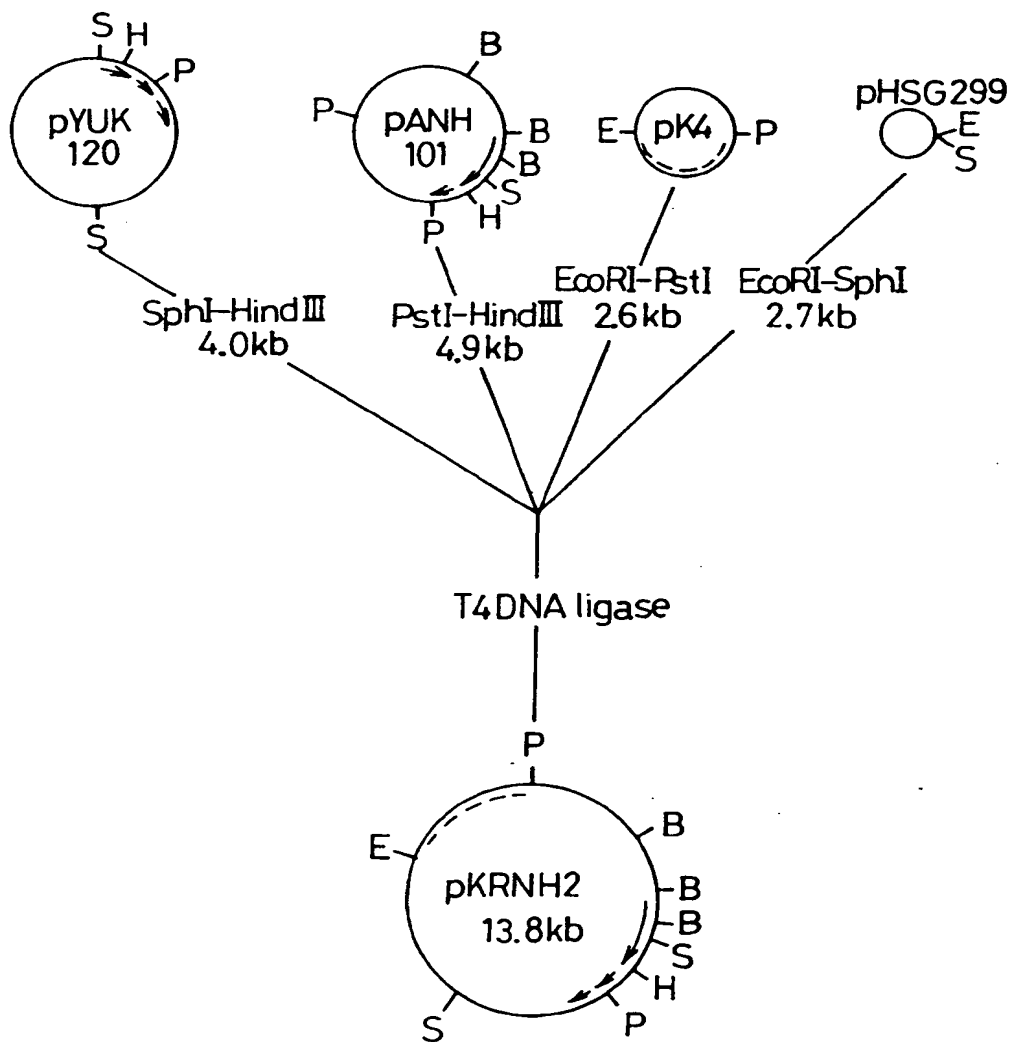
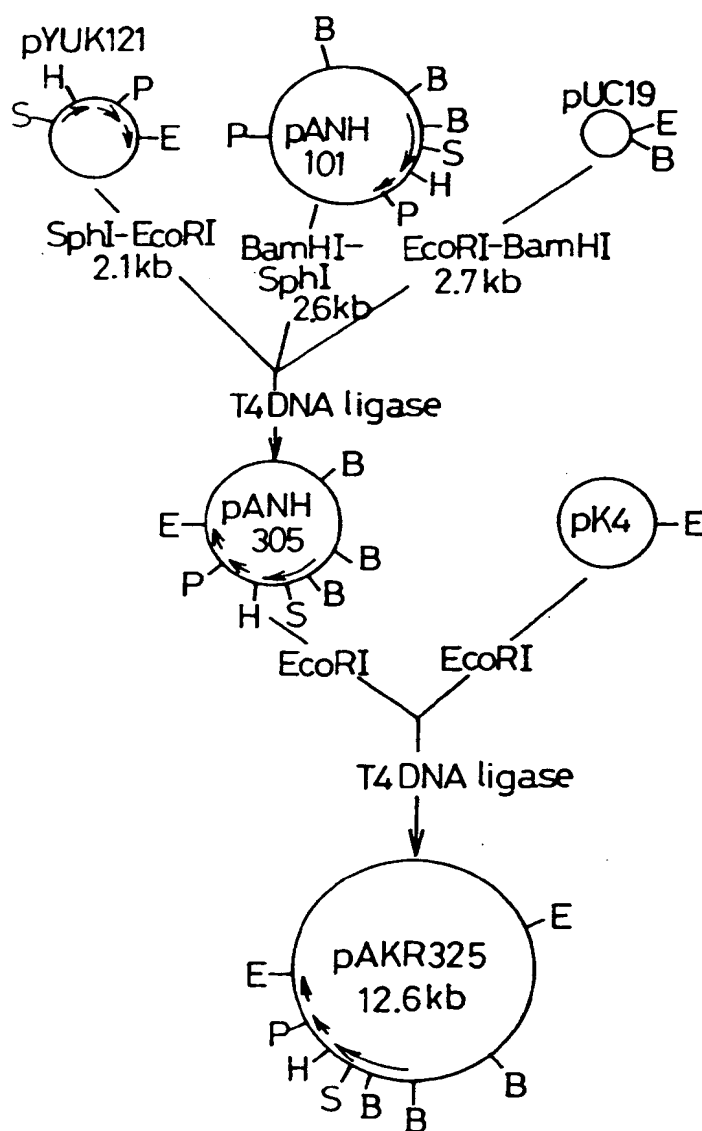


Fig. 5



THIS PAGE BLANK (USPTO)

Fig. 1

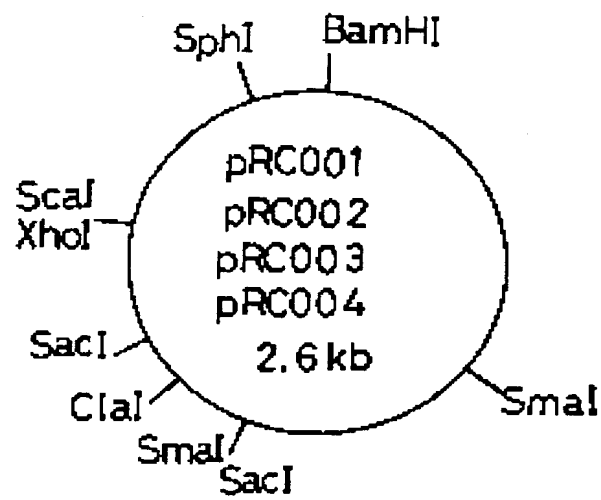


Fig. 2

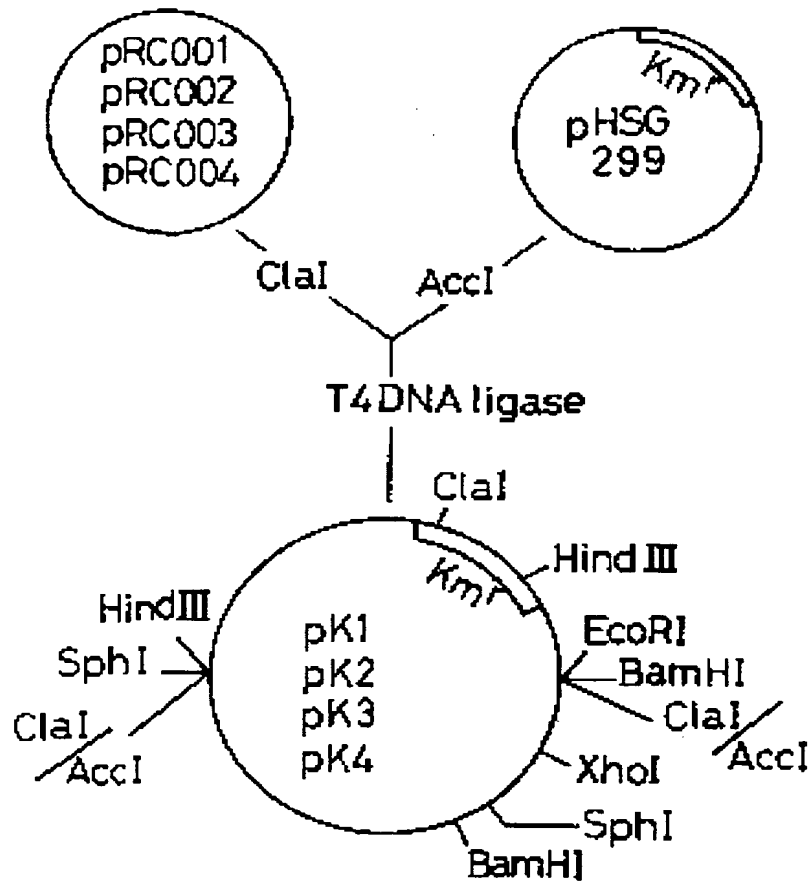


Fig. 3

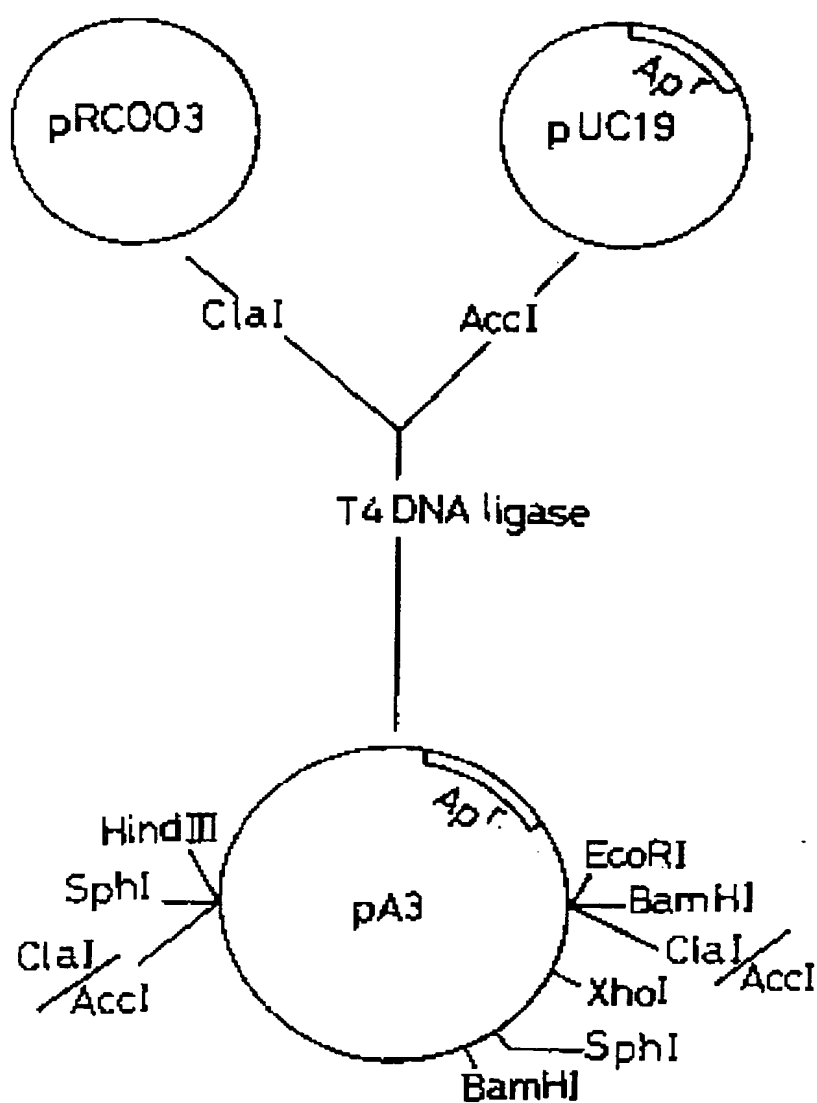


Fig.4

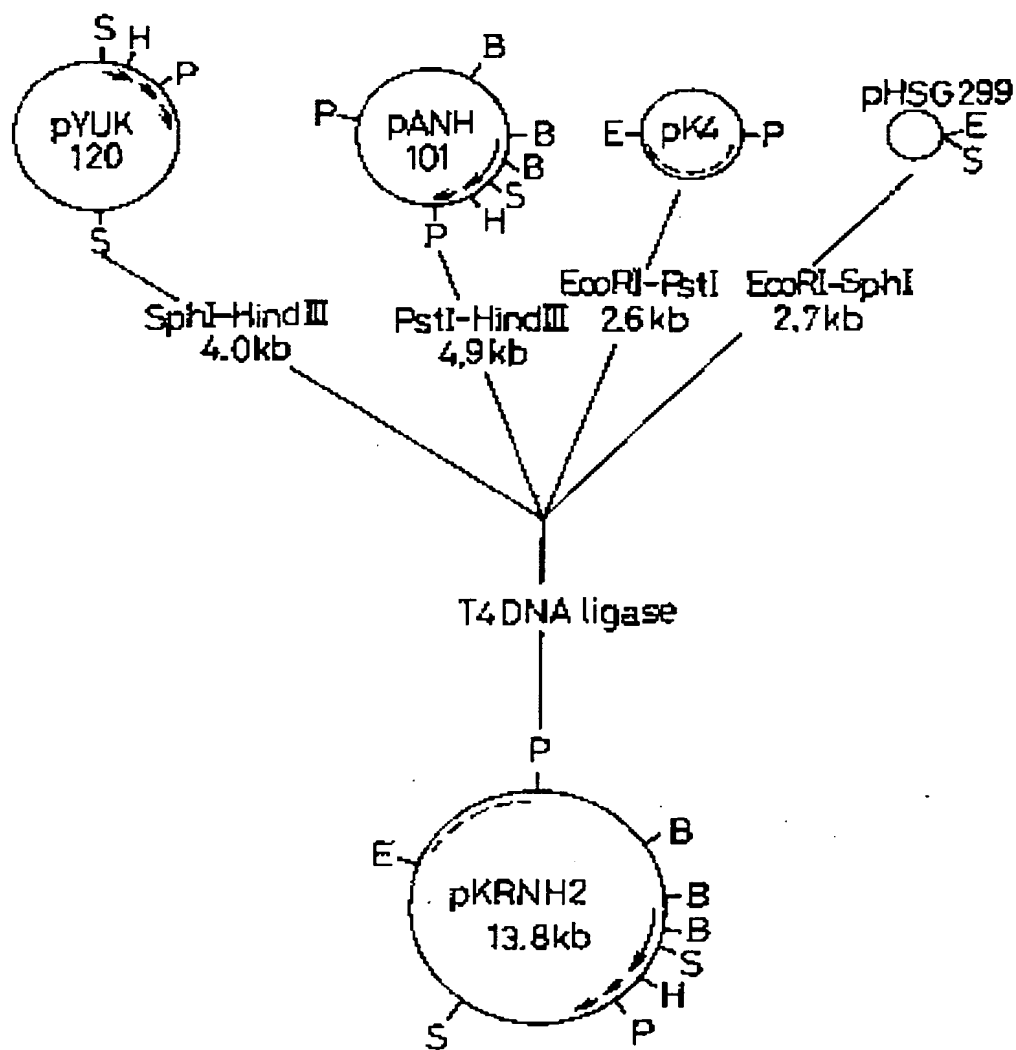
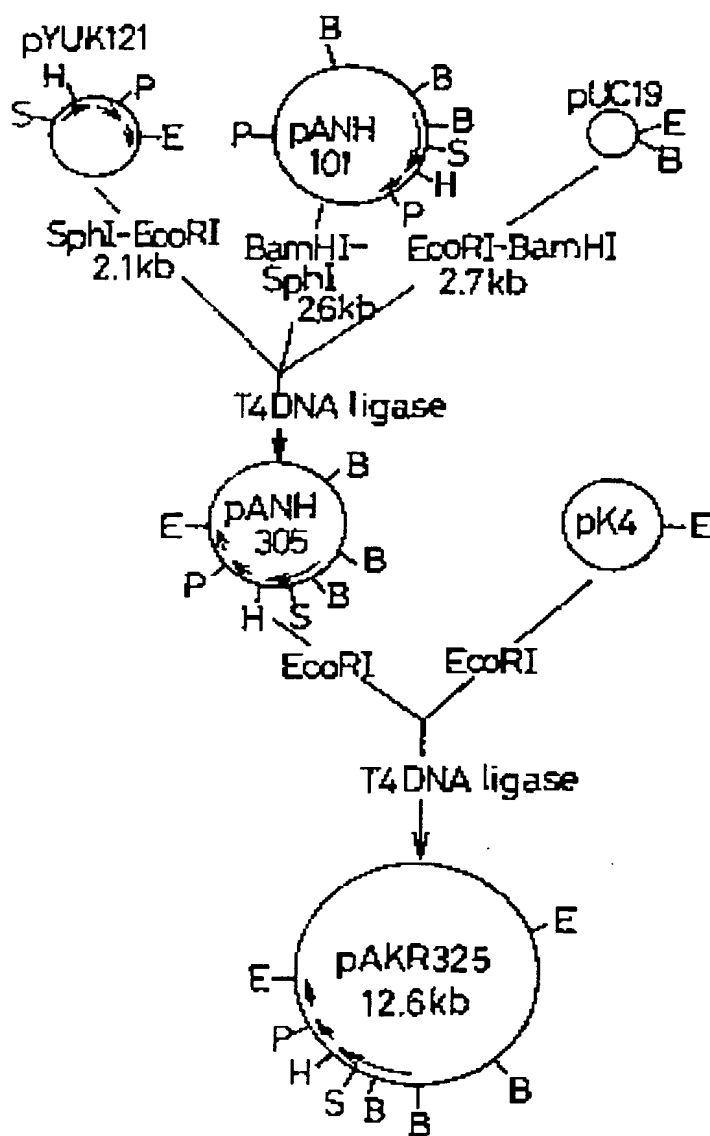


Fig. 5





Europäisches Patentamt
European Patent Office
Office européen des brevets



Publication number: **0 502 476 A3**

12

EUROPEAN PATENT APPLICATION

Application number: **92103610.9**

Date of filing: **03.03.92**

Int. Cl.⁵: **C12N 15/74, C12N 15/70,
C12N 15/55, C12N 1/21,
/(C12N1/21,C12R1:01)**

Priority: **04.03.91 JP 37544/91
04.03.91 JP 37545/91**

Date of publication of application:
09.09.92 Bulletin 92/37

Designated Contracting States:
DE FR GB

Date of deferred publication of the search report:
21.04.93 Bulletin 93/16

Applicant: **NITTO CHEMICAL INDUSTRY CO.,
LTD.**

**No. 5-1, Marunouchi 1-chome Chiyoda-ku
Tokyo(JP)**

Applicant: **Beppu, Teruhiko**

**No. 5-21, 1-chome
Horinouchi Suginami-ku, Tokyo(JP)**

Inventor: **Beppu, Teruhiko
5-21 Horinouchi 1-chome, Suginami-ku
Tokyo(JP)**

Inventor: **Horinouchi, Sueharu
3-16-403, Etsuchujima 1-chome
Koutou-ku, Tokyo(JP)**

Inventor: **Nishiyama, Makoto
16-11, Nishiokhlal 2-chome
Shinjuku-ku, Tokyo(JP)**

Inventor: **Yu, Fujio
24-4-108, Kirigaoka 3-chome, Midori-ku
Yokohama-shi, Kanagawa-ken(JP)**
Inventor: **Hashimoto, Yoshihiro
8-2-104, Yayoi 2-chome, Bunkyo-ku
Tokyo(jp)**

Representative: **Vossius & Partner
Siebertstrasse 4 P.O. Box 86 07 67
W-8000 München 86 (DE)**

Hybrid plasmid vectors containing genes encoding nitrile degrading enzymes and methods of producing amides and acids.

The present invention provides a recombinant plasmid comprising combining a hybrid plasmid vector with the isolated DNA sequences of one or more genes encoding nitrile degrading enzymes which are derived from bacteria belonging to the genus *Rhodococcus*, said hybrid plasmid vector comprising an isolated DNA sequence which confers the vector the ability to replicate and amplify in the cells of bacteria belonging to the genus *Rhodococcus*, and an isolated DNA sequence which confers the vector

the ability to replicate and amplify in the cells of bacteria belonging to *Escherichia coli*, and an isolated DNA sequence containing a drug resistance gene.

EP 0 502 476 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 10 3610

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	US-A-4 920 054 (KOZLOWSKI ET AL.) 24 April 1990 * the whole document * ---	7,10-13	C12N15/74 C12N15/70 C12N15/55 C12N1/21 // (C12N1/21, C12R1/01)
Y	WO-A-8 907 151 (UNIVERSITY OF GERGIA RESEARCH FOUNDATION, INC.) 10 August 1989 * the whole document * ---	7,10-13	
Y	FR-A-2 633 938 (TERUHIKO BEPPU ET NITTO CHEMICAL INDUSTRY CO. LTD) 12 January 1990 * the whole document * ---	7,10-13	
P,Y	EP-A-0 445 646 (NITTO CHEMICAL INDUSTRY CO., LTD) 11 September 1991 * the whole document * -----	7,10-13	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C12N
Place of search THE HAGUE		Date of completion of the search 24 FEBRUARY 1993	Examiner HORNIG H.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

EPO FORM 1503 (3.12.1990)